

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/18, C07K 14/16, 14/155, A61K 48/00		A2	(11) International Publication Number: WO 95/24485 (43) International Publication Date: 14 September 1995 (14.09.95)
<p>(21) International Application Number: PCT/US95/02633</p> <p>(22) International Filing Date: 3 March 1995 (03.03.95)</p> <p>(30) Priority Data: 207,526 7 March 1994 (07.03.94) US</p> <p>(60) Parent Application or Grant (63) Related by Continuation US 207,526 (CIP) Filed on 7 March 1994 (07.03.94)</p> <p>(71) Applicant (for all designated States except US): MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): LIU, Margaret, A. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). SHIVER, John, W. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). PERRY, Helen, C. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).</p>		<p>(74) Common Representative: MERCK & CO., INC.; Patent Dept., 126 East Lincoln Avenue, Rahway, NJ 07065 (US).</p> <p>(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, JP, KG, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TT, UA, US, UZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).</p> <p>Published Without international search report and to be republished upon receipt of that report.</p>	
<p>(54) Title: COORDINATE IN VIVO GENE EXPRESSION</p> <p>tPA-gp120 (V1 Jns-tPA-gp120)</p> <pre> graph LR tPA[tPA LEADER PEPTIDE] --- gp120[gp120] gp120 --- IRES[IRES] IRES --- B7[B7] </pre> <p>gp160/rev DICISTRONIC CONSTRUCT (V1 Jns-gp160/IRES/rev/SD)</p> <pre> graph TD tPA[tPA LEADER PEPTIDE] --- gp120[gp120] gp120 --- gp41[gp41] gp41 --- IRES[IRES] IRES --- rev[rev] RRE[RRE] --- gp120 RRE --- gp41 RRE --- IRES RRE --- rev </pre> <p><u>HIV gag/rev DICISTRONIC CONSTRUCT SCHEMATIC</u></p> <pre> graph LR p55[p55 gag] --- RRE[RRE] RRE --- IRES[IRES] IRES --- rev[rev] </pre> <p>(57) Abstract</p> <p>Nucleic acids, including DNA constructs and RNA transcripts, capable of inducing coordinate expression of two to three cistrons upon direct introduction into animal tissues, are presented. Bi- or tri-cistronic polynucleotides of this invention include those encoding and co-expressing HIV gene products, genes encoding antigens unrelated to HIV, and immunostimulatory gene products, including but not limited to GM-CSF, interleukins, interferon and members of the B7 family of proteins which act as T-cell costimulatory elements. The methods and polynucleotides of this invention are generally applicable to co-ordinate expression <i>in vivo</i> of any two or more genes in a single cell.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

- 1 -

TITLE OF THE INVENTION

COORDINATE IN VIVO GENE EXPRESSION

CROSS-RELATED TO OTHER APPLICATIONS

5 This is a continuation-in-part of U. S. Serial Number 08/207,526, filed March 7, 1994, now pending.

BACKGROUND OF THE INVENTION

10 1. Field of the Invention

A method for coordinate expression in a single cell, in vivo, of exogenous genes via introduction into the tissue of a vertebrate of polycistronic polynucleotide constructs is described. The method results in production of immune responses against the products produced as a result of expression of the exogenous genes. The method and polynucleotide constructs of this invention may be used in a vertebrate to generate immune responses against antigenic epitopes expressed by a single cell. The coordinate expression results in improved expression of gene products which may be otherwise poorly expressed. It also results in improved cellular immune responses due to provision of T-cell stimulatory signals by the same cell expressing T-cell antigens. Polynucleotide constructs encoding human immunodeficiency virus (HIV) antigens exemplify one embodiment of the method.

25 2. Background of the Invention

A major challenge to the development of vaccines against viruses, particularly viruses with a high rate of mutation such as HIV, against which elicitation of neutralizing and protective immune responses is desirable, is the diversity of the viral envelope proteins among different viral isolates or strains. Because cytotoxic T-lymphocytes (CTLs) in both mice and humans are capable of recognizing epitopes derived from conserved internal viral proteins and may be important in the immune response against viruses, efforts have

- 2 -

been directed towards the development of CTL vaccines that elicit heterologous protection against different viral strains.

CD8⁺ CTLs kill virally-infected cells when their T cell receptors recognize viral peptides associated with MHC class I molecules. These peptides are derived from endogenously synthesized viral proteins. Thus, by recognition of epitopes from conserved viral proteins, CTLs may provide cross-strain protection. Peptides capable of associating with MHC class I for CTL recognition originate from proteins that are present in or pass through the cytoplasm or endoplasmic reticulum. Exogenous proteins which enter the endosomal processing pathway (as in the case of antigens presented by MHC class II molecules) are not usually effective in generating CD8⁺ CTL responses.

Efforts to generate CTL responses have used replicating vectors to produce the protein antigen within the cell or have introduced peptides into the cytosol. These approaches have limitations that may limit their utility as vaccines. Retroviral vectors have restrictions on the size and structure of polypeptides that can be expressed as fusion proteins while maintaining the ability of the recombinant virus to replicate. Further, the effectiveness of vectors such as vaccinia for subsequent immunizations may be compromised by immune responses against the vectors themselves. Also, viral vectors and modified pathogens have inherent risks that may hinder their use in humans [R.R. Redfield *et al.*, New Engl. J. Med. **316**, 673 (1987); L. Mascola *et al.*, Arch. Intern. Med. **149**, 1569 (1989)]. Furthermore, the selection of peptide epitopes to be presented is dependent upon the structure of an individual's MHC antigens; thus, peptide vaccines may have limited effectiveness due to the diversity of MHC haplotypes in outbred populations.

Benvenisty, N., and Reshef, L. [PNAS **83**, 9551-9555, (1986)] showed that CaCl₂-precipitated DNA introduced into mice intraperitoneally (i.p.), intravenously (i.v.) or intramuscularly (i.m.) could be expressed. Intramuscular injection of DNA expression vectors in mice results in the uptake of DNA by the muscle cells and expression of the protein encoded by the DNA [J.A. Wolff *et al.*, Science **247**,

1465 (1990); G. Ascadi *et al.*, *Nature* **352**, 815 (1991)]. The plasmids were maintained episomally and did not replicate. Subsequently, persistent expression has been observed after i.m. injection in skeletal muscle of rats, fish and primates, and cardiac muscle of rats. The technique of using nucleic acids as therapeutic agents was reported in WO90/11092 (4 October 1990), in which naked polynucleotides were used to vaccinate vertebrates.

It is not necessary for the success of the method that immunization be intramuscular. Thus, Tang *et al.*, [Nature, **356**, 152-154 (1992)] disclosed that introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of mice resulted in production of anti-BGH antibodies in the mice. Furth *et al.*, [Anal. Biochem. **205**, 365-368, (1992)] showed that a jet injector could be used to transfect skin, muscle, fat, and mammary tissues of living animals. Methods for introducing nucleic acids was recently reviewed by Friedman, T., [Science, **244**, 1275-1281 (1989)]. Robinson *et al.*, [Abstracts of Papers Presented at the 1992 meeting on Modern Approaches to New Vaccines, Including Prevention of AIDS, Cold Spring Harbor, p92] reported that i.m., i.p., and i.v. administration of avian influenza DNA into chickens provided protection against lethal challenge. However, Robinson *et al.* did not disclose which avian influenza virus genes were used. In addition, only H7 specific immune responses were alleged; the induction of cross-strain protection was not discussed. Intravenous injection of a DNA:cationic liposome complex in mice was shown by Zhu *et al.*, [Science **261**:209-211 (9 July 1993); see also WO93/24640, 9 Dec. 1993] to result in systemic expression of a cloned transgene. Recently, Ulmer *et al.*, [Science **259**:1745-1749, (1993)] reported on the heterologous protection against influenza virus infection by injection of DNA encoding influenza virus proteins.

The need for specific therapeutic and prophylactic agents capable of eliciting desired immune responses against pathogens and tumor antigens is achieved by the instant invention. Of particular importance in this therapeutic approach is the ability to induce T-cell

- 4 -

immune responses which can prevent infections or disease caused by virus strains which are heterologous to the strain from which the antigen gene was obtained. This is of significance with HIV, since HIV mutates rapidly, and because many virulent isolates have been identified [see, for example, LaRosa et al., *Science* 249:932-935 (1990), identifying 245 separate HIV isolates].

In response to this diversity, researchers have attempted to generate CTLs by peptide immunization. Thus, Takahashi et al., [Science 255:333-336 (1992)] reported on the induction of broadly cross-reactive cytotoxic T cells recognizing an HIV envelope (gp160) determinant. They recognized the difficulty in achieving a truly cross-reactive CTL response and suggested that there is a dichotomy between the priming or restimulation of T cells, which is very stringent, and the elicitation of effector function, including cytotoxicity, from already stimulated CTLs.

Wang et al., [P.N.A.S. USA 90:4156-4160 (May, 1993)] reported on elicitation of immune responses in mice against HIV by intramuscular inoculation with a cloned, genomic (unspliced) HIV gene. The level of immune response achieved was low, and the system utilized portions of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) promoter and portions of the simian virus 40 (SV40) promoter and terminator. SV40 is known to transform cells, possibly through integration into host cellular DNA. Therefore, unlike the system described herein, the system described by Wang et al. may be inappropriate for administration to humans. In addition, the DNA construct of Wang et al. contains an essentially genomic piece of HIV encoding contiguous Tat/REV-gp160-Tat/REV coding sequences (Figure 1). As is described in detail below, this is a suboptimal system for obtaining high-level expression of the gp160. One drawback is that the expression of Tat has been recognized to play a contributory role in the progression of Kaposi's Sarcoma, [Y.N. Vaishav and F.W. Wong-Staal, *An. Rev. Biochem.* (1991)].

WO 93/17706 describes a method for vaccinating an animal against a virus, wherein carrier particles were coated with a gene

construct and the coated particles are accelerated into cells of an animal. In regard to HIV, essentially the entire genome, minus the long terminal repeats, was proposed to be used. That method may represent a substantial risk for recipients. Constructs of HIV should, in general, contain less than about 50% of the HIV genome to ensure safety of the vaccine. Thus, a number of problems remain if a useful human HIV vaccine is to emerge from the gene-delivery technology.

The instant invention uses known methods for introducing polynucleotides into living tissue to induce expression of proteins. This invention provides a immunogen for introducing HIV and other proteins into the antigen processing pathway to efficiently generate HIV-specific CTLs and antibodies. The pharmaceutical is effective as a vaccine to induce both cellular and humoral anti-HIV and HIV neutralizing immune responses. The instant invention addresses some of the problems by providing polynucleotide immunogens which, when introduced into an animal, direct the efficient expression of HIV proteins and epitopes without the attendant risks associated with those methods. The immune responses generated are effective at recognizing HIV, at inhibiting replication of HIV, at identifying and killing cells infected with HIV, and are cross-reactive against many HIV strains. Therefore, this invention provides a useful immunogen against HIV. The invention also provides polynucleotide constructs which enable the co-expression, in vivo, of more than one gene-product in a single cell. This is demonstrated with an HIV gene expression system in which the expression of a first gene is dependent on the co-expression in the same cell of a second gene product. By virtue of the success of achieving this co-expression in vivo, it is now predictable that this type of polynucleotide construct may be applied to co-expression in vivo of many combinations of gene products, including but not limited to viral antigens other than HIV related antigens, carcinoma-associated antigens, and immunomodulatory or immunostimulatory gene products.

SUMMARY OF THE INVENTION

Nucleic acids, including DNA constructs and RNA transcripts, capable of inducing coordinate expression of two to three cistrons upon direct introduction into animal tissues, are presented. In one embodiment, coordinate expression of two cistrons encoding HIV proteins and elicitation of HIV specific immune responses against more than one gene products is demonstrated. Cytotoxic T lymphocytes (CTLs) specific for viral antigens which respond to different strains of human immunodeficiency virus (HIV), and antibodies which are generally strain-specific are generated. The generation of such CTLs in vivo usually requires endogenous expression of the antigen, as in the case of virus infection. To generate a viral antigen for presentation to the immune system, without the limitations of direct peptide delivery or the use of viral vectors, polynucleotides encoding HIV proteins are directly introduced into tissues of vertebrates in vivo, the polynucleotides are taken up by cells within the tissue, and the encoded proteins produced and processed for presentation to the immune system. In mice, this resulted in the generation of HIV-specific CTLs and antibodies. Similar results are achieved in primates. These results are achieved with bi- or tri-cistronic nucleic acid polynucleotides encoding and co-expressing HIV gene products, immunostimulatory gene products including but not limited to GM-CSF, interleukins, interferon and B7 proteins, which act as T-cell costimulatory elements. The methods and polynucleotides of this invention are generally applicable to co-ordinate expression in vivo of any two or three genes. Thus, various embodiments of this invention include coordinate expression in vivo of viral antigens and immunostimulatory gene products as well as coordinate expression of tumor antigens and immunostimulatory genes.

30

BRIEF DESCRIPTION OF THE DRAWINGS

- Fig. 1. A schematic representation of the HIV genome.
- Fig. 2. A schematic representation of a polynucleotide construct of this invention capable of inducing the co-ordinate expression in vivo in a single cell of up to three gene products encoded

- 7 -

by each of three cistrons (I, II, and III). The segments A and B represent control sequences including transcription termination signals and promoters or internal ribosome entry sites (IRES).

5 Fig. 3. Detailed schematic of an HIV env polynucleotide immunogen construct comprising the CMV-intA transcription promoter, a 5'-splice donor, HIV gp160 (showing gp120, gp41, and the REV-responsive element, RRE), an internal ribosome entry site (IRES), the REV cistron, the BGH transcription terminator, and the neomycin resistance marker which is driven by a prokaryotic transcription promoter..

10 Fig. 4. Detailed schematic of dicistronic HIV env and gag polynucleotide immunogen constructs showing specific regulatory elements.

15 Fig. 5. Western blot analysis of gp160 expression induced by HIV polynucleotide immunogens. This result rigorously shows the coexpression in a single cell of more than one gene product from a single polynucleotide construct: A polynucleotide encoding gp160 alone (see panel B, fourth lane from the left) expresses no detectable gp160, but with REV added in trans (by cotransfection of a construct encoding only REV), there is good gp160 expression (panel A, fourth lane from the left). A genomic tat/REV/env construct expresses only low levels of gp160, whether or not REV is provided in trans (panels A and B, third lane). However, a dicistronic gp160/IRES/REV construct heavily expresses gp160 (panels A and B, fifth lane from the left). The best expression, is obtained in a dicistronic construct encoding gp160/IRES/REV, with a splice donor (SD) provided 5' to the gp160 coding sequence (panels A and B, right hand lane). Because no additional expression is achieved when additional REV is provided in trans (panel A right hand lane), the system is not limited by the level of REV being expressed.

20 25 30 Fig. 6. V1J Sequence.

- Fig. 7. V1Jneo Sequence.
- Fig. 8. CMVintABGH Sequence.
- Fig. 9. Cytotoxic T lymphocytes generated in rhesus monkeys in response to V1J-SIV-p28 polynucleotide construct vaccination (REV independent). This SIV p28 is equivalent to p24 gag of HIV. Thus, CTLs specific to a group specific antigen are inducible using a gag encoding polynucleotide construct.
- Fig.10. Cytotoxic T lymphocytes generated in response to Vaccinia-SIVp28 nucleic acid vaccination. This demonstrates that similar CTLs are induced by a gag encoding polynucleotide (figure 9) as compared with a replicating antigen (vaccinia) expressing the same antigen [see Shen, L., et al., Science 252:440-443, 1991].
- Fig.11. Sequence of the Vector VIR.
- Fig.12. Antibodies induced by V1Jns-tPA-gp120, 200 µg/mouse per round, 2 rounds.
- Fig.13. Neutralization of HIV-1 (MN) virus by sera from V1Jns-tPA-gp120 (MN) DNA vaccinated African Green Monkeys. Panels a and B show the reduction in p24 gag protein production for C8166 cells infected with HIV-1 (MN) following exposure to the indicated dilutions of sera from V1Jns-tPA-gp120 DNA vaccinated monkeys. Data was obtained after 10 days in tissue culture following virus inoculation (TCID50 per sample).
- Fig.14 T cells from V1Jns-tPA-gp120 vaccinated mice exhibiting long-term, antigen-specific T lymphocyte memory responses. Immunized mice received 1.6 mcg of vaccine DNA twice, six months prior to sacrifice. Splenic T cells were cultured in vitro with recombinant gp120 protein at 5 mcg/mL. Proliferation of gp120-specific T cells. A stimulation index (SI; incorporated ³H-thymidine for gp120 treated T cells:T cells that did not receive antigen).
- Fig. 15. Type 1 T helper (TH1) lymphocyte cytokine secretion by T cells from V1Jns-tPA-gp120 DNA vaccinated mice. Cell culture supernatants from the samples shown in Figure 13 were assayed for gamma-interferon and interleukin 4 (IL-4) secretion following

- 9 -

treatment with rgp120. Immune mice secreted large amounts of gamma-interferon and very low amounts of IL-4 indicated that TH1-like responses were induced by this vaccine. Control mice showed very low amounts of interferon secretion while the IL-4 levels indicated are background levels.

Fig. 15 Anti-gp120 cytotoxic T lymphocyte (CTL) activities in V1Jns-tPA-gp120 DNA vaccinated mice. Two mice (2006 and 2008) showed MHC I restricted CTL activities specific to a gp120 peptide (P18) following gp120 DNA vaccinations. No activities were observed for these mice in the absence of P18 or by a control mouse which had not been previously vaccinated.

Fig. 16. Anti-gp160 CTL activities by rhesus monkeys vaccinated with V1Jns-gp160/IRES/rev and V1Jns-tPA-gp120 DNA vaccines. T lymphocyte cultures from all four monkeys receiving these vaccines showed MHC I restricted killing of autologous target cells that had been treated with vaccinia-gp160. No CTL activity was observed in four control rhesus that had been immunized with 'blank' DNA vaccine (V1Jns without a gene insert).

20 DETAILED DESCRIPTION OF THE INVENTION

Nucleic acids, including DNA constructs and RNA transcripts, capable of inducing coordinate expression of two to three cistrons upon direct introduction into animal tissues, are presented. In one embodiment, coordinate expression of two cistrons encoding HIV proteins and elicitation of HIV specific immune responses against more than one gene products is demonstrated. Cytotoxic T lymphocytes (CTLs) specific for viral antigens which respond to different strains of human immunodeficiency virus (HIV), and antibodies which are generally strain-specific are generated. The generation of such CTLs in vivo usually requires endogenous expression of the antigen, as in the case of virus infection. To generate a viral antigen for presentation to the immune system, without the limitations of direct peptide delivery or the use of viral vectors, polynucleotides encoding HIV proteins are directly introduced into tissues of vertebrates in vivo, the

polynucleotides are taken up by cells within the tissue, and the encoded proteins produced and processed for presentation to the immune system. In mice, this resulted in the generation of HIV-specific CTLs and antibodies. Similar results are achieved in primates. These results are achieved with bi- or tri-cistronic nucleic acid polynucleotides encoding and co-expressing HIV gene products, immunostimulatory gene products including but not limited to GM-CSF, interleukins, interferon and B7 proteins, which act as T-cell costimulatory elements. The methods and polynucleotides of this invention are generally applicable to co-ordinate expression in vivo of any two or three genes. Thus, various embodiments of this invention include coordinate expression in vivo of viral antigens and immunostimulatory gene products as well as coordinate expression of tumor antigens and immunostimulatory genes.

This invention provides polynucleotides which, when directly introduced into a vertebrate in vivo, including mammals such as primates and humans, induces the expression of encoded proteins within the animal.

As used herein, a polynucleotide is a nucleic acid which contains essential regulatory elements such that upon introduction into a living vertebrate cell, is able to direct the cellular machinery to produce translation products encoded by the genes comprising the polynucleotide.

In one embodiment of the invention, the polynucleotide is a polydeoxyribonucleic acid comprising HIV genes operatively linked to a transcriptional promoter. In another embodiment of the invention, the polynucleotide vaccine comprises polyribonucleic acid encoding HIV genes which are amenable to translation by the eukaryotic cellular machinery (ribosomes, tRNAs, and other translation factors). Where the protein encoded by the polynucleotide is one which does not normally occur in that animal except in pathological conditions, (i.e. an heterologous protein) such as proteins associated with human immunodeficiency virus, (HIV), the etiologic agent of acquired immune deficiency syndrome, (AIDS), the animals' immune system is activated to launch a protective immune response. Because these exogenous

proteins are produced by the animals' own tissues, the expressed proteins are processed by the major histocompatibility system, MHC, in a fashion analogous to when an actual infection with the related organism, HIV, occurs. The result, as shown in this disclosure, is induction of immune responses against the cognate pathogen.

Accordingly, the instant inventors have prepared nucleic acids which, when introduced into the biological system induce the expression of HIV proteins and epitopes. The induced antibody response is both specific for the expressed HIV protein, and neutralizes HIV. In addition, cytotoxic T-lymphocytes which specifically recognize and destroy HIV infected cells are induced. The instant inventors have also developed polynucleotides whereby simian immunodeficiency virus (SIV) genes are efficiently expressed upon introduction in vivo. This achievement is significant because the only animal model closely mimicking the human disease, AIDS, is the subhuman primate model utilizing SIV. Thus, efficacy of the instant immunogens as vaccines can be shown by analogy to the effects obtained in vivo utilizing HIV and SIV polynucleotide immunogens.

There are many embodiments of the instant invention which those skilled in the art can appreciate from the specifics taught herein. Thus, different transcriptional promoters, terminators, carrier vectors or specific gene sequences may be used successfully based on the successful invention disclosed herein.

The instant invention provides a method for using a polynucleotide which, upon introduction into mammalian tissue, induces the co-expression in a single cell, in vivo, of two or more different, discrete gene products. The method is exemplified by using an HIV model which demonstrates the co-expression of more than one gene product in a single cell upon introduction of the polynucleotide into mammalian tissue in vivo. The model is stringent because certain HIV genes contain a sequence known as the REV responsive element (RRE). These genes are not efficiently expressed unless another HIV gene, known as REV, is also present within the cell expressing the RRE-

containing HIV gene. This phenomenon is described as REV dependence.

5 Pavlakis and Felber, WO 93/20212 have described a method of eliminating sequences which may induce transcript instability, which may also achieve some REV independence of certain HIV genes. That method may not be generally applicable to all such genes, is time-consuming and may require multiple gene modifications. Furthermore, the level of expression and immunogenicity of such genes 10 may be compromised by elimination of the REV dependence.

The instant invention provides a different solution which does not require multiple manipulations of REV dependent HIV genes to obtain REV-independence. In addition, the instant invention is applicable to expression of REV independent genes as well as to expression of REV dependent genes. The REV-dependent expression 15 system described herein, is useful in its own right and is also useful as a stringent system for demonstrating the co-expression in a single cell in vivo of more than a single desired gene-product. Thus, in any circumstance in which it is beneficial to achieve the co-expression, 20 within a given cell in vivo, of more than a single gene product, the methods and polynucleotide constructs described herein may be employed.

One situation, exemplified herein, is the co-expression of 25 an immunogenic epitope and a member of the family of T-cell recognition elements known as B7. Recently, Steven Edgington [Biotechnology 11:1117-1119, 1993] reviewed the coordinate roles of B7 and the major histocompatibility complex (MHC) presentation of epitopes on the surface of antigen presenting cells in activating CD8⁺ CTLs for the elimination of tumors. Once a MHC molecule on the 30 surface of an antigen presenting cell (APC) presents an epitope to a T-cell receptor (TCR), B7 expressed on the surface of the same APC acts as a second signal by binding to CTLA-4 or CD28. The result is rapid division of CD4⁺ helper T-cells which signal CD8⁺ T-cells to proliferate and kill the APC. Thus, our demonstration herein of efficient expression and production of immune responses against an HIV

REV dependent gene containing an RRE by coordinately expressing a gene for REV, conclusively proves that more than one gene can be coordinately expressed by introducing a polynucleotide encoding two and even three cistrons (defined as a stretch of nucleic acid that carries the information for a polypeptide chain).

Because many of the applications of the instant invention apply to anti-viral vaccination, the polynucleotides are frequently referred to as a polynucleotide vaccine (PNV). This is not to say that additional utilities of these polynucleotides, in immune stimulation and in anti-tumor therapeutics, is to be ignored or considered to be outside the scope of the invention.

In one embodiment of this invention, a gene encoding an HIV gene product is incorporated in an expression vector. The vector contains a transcriptional promoter recognized by an eukaryotic RNA polymerase, and a transcriptional terminator at the end of the HIV gene coding sequence. In a preferred embodiment, the promoter is the cytomegalovirus promoter with the intron A sequence (CMV-intA), although those skilled in the art will recognize that any of a number of other known promoters such as the strong immunoglobulin, or other eukaryotic gene promoters may be used. A preferred transcriptional terminator is the bovine growth hormone terminator. The combination of CMVintA-BGH terminator (Fig. 8, SEQ. ID:13:) is particularly preferred. In addition, to assist in preparation of the polynucleotides in prokaryotic cells, an antibiotic resistance marker is also preferably included in the expression vector under transcriptional control of a prokaryotic promoter so that expression of the antibiotic does not occur in eukaryotic cells. Ampicillin resistance genes, neomycin resistance genes or any other pharmaceutically acceptable antibiotic resistance marker may be used. In a preferred embodiment of this invention, the antibiotic resistance gene encodes a gene product for neomycin resistance. Further, to aid in the high level production of the polynucleotide by fermentation in prokaryotic organisms, it is advantageous for the vector to contain a prokaryotic origin of replication and be of high copy number. Any of a number of

commercially available prokaryotic cloning vectors provide these benefits. In a preferred embodiment of this invention, these functionalities are provided by the commercially available vectors known as pUC. It is desirable, however, to remove non-essential DNA sequences. Thus, the lacZ and lacI coding sequences of pUC are removed in one embodiment of the invention. It is also desirable that the vectors not be able to replicate in eukaryotic cells. This minimizes the risk of integration of polynucleotide vaccine sequences into the recipients' genome.

In another embodiment, the expression vector pnRSV is used, wherein the Rous Sarcoma virus (RSV) long terminal repeat (LTR) is used as the promoter. In yet another embodiment, V1, a mutated pBR322 vector into which the CMV promoter and the BGH transcriptional terminator were cloned is used. In a particularly preferred embodiment of this invention, the elements of V1 and pUC19 have been combined to produce an expression vector named V1J (SEQ. ID:12:). Into V1J or another desirable expression vector is cloned an HIV gene, such as gp120, gp41, gp160, gag, pol, env, or any other HIV gene which can induce anti-HIV immune responses (antibody and/or CTLs). Exclusion of functional reverse transcriptase and integrase functions encoded by the HIV genome is desirable to minimize the risk of integration of the polynucleotide vaccine encoded sequences into the recipients' genome. In another embodiment, the ampicillin resistance gene is removed from V1J and replaced with a neomycin resistance gene, to generate V1J-neo (SEQ.ID:14:), into which any of a number of different HIV genes have been cloned for use according to this invention. In yet another embodiment, the vector is V1Jns, which is the same as V1Jneo except that a unique Sfi1 restriction site has been engineered into the single Kpn1 site at position 2114 of V1J-neo. The incidence of Sfi1 sites in human genomic DNA is very low (approximately 1 site per 100,000 bases). Thus, this vector allows careful monitoring for expression vector integration into host DNA, simply by Sfi1 digestion of extracted genomic DNA. In a further refinement, the vector is V1R. In this vector, as much non-essential

DNA as possible was "trimmed" from the vector to produce a highly compact vector. This vector is a derivative of V1Jns and is shown in Figure 11, (SEQ.ID.:100:). This vector allows larger inserts to be used, with less concern that undesirable sequences are encoded and optimizes uptake by cells when the construct encoding specific influenza virus genes is introduced into surrounding tissue. In figure 11, the portions of V1Jneo (Figure 7) that are deleted are shown as a gap, and inserted sequence is in bold text, but the numbering of V1Jneo is unchanged.

The foregoing vector modification and development procedures may be accomplished according to methods known by those skilled in the art. The particular products described however, though obtained by conventional means, are especially useful for the particular purpose to which they are adapted.

One embodiment of this invention incorporates genes encoding HIV gp160, gp120, gag and other gene products from such well known laboratory adapted strains of HIV as SF2, IIIB or MN, for which a great deal of data has been generated, for example, such as showing that chimpanzees can be protected from a lethal challenge of HIV IIIB virus by first administering HIV IIIb V3 loop specific monoclonal antibody [Emini et al., *Nature* 355: 728-730 1992], or by vaccination with recombinant gp120 but not gp160 [Berman et al., *Nature* 345 : 822-825, 1990]. Those skilled in the art will recognize that the use of genes from HIV-2 strains having analogous function to the genes from HIV-1 would be expected to generate immune responses analogous to those described herein for HIV-1 constructs. The cloning and manipulation methods for obtaining these genes are well known to those skilled in the art.

There has recently been recognition that elicitation of immune responses against laboratory adapted strains of HIV may not be adequate to provide neutralization of primary, field isolates of HIV, [see for example Cohen, J., *Science* 262: 980-981, 1993]. Thus, in another embodiment of this invention, genes from virulent, primary field isolates of HIV are incorporated in the polynucleotide immunogen. This is accomplished by preparing cDNA copies of the viral genes and

then subcloning the individual genes into the polynucleotide immunogen. Sequences for many genes of many HIV strains are now publicly available on GENBANK and such primary, filed isolates of HIV are available from the National Institute of Allergy and Infectious Diseases (NIAID) which has contracted with Quality Biological, Inc., [7581 Lindbergh Drive, Gaithersburg, Maryland 20879] to make these strains available. Such strains are also now available from the World Health Organization (WHO) [Network for HIV Isolation and Characterization, Vaccine Development Unit, Office of Research, Global Program on AIDS, CH-1211 Geneva 27, Switzerland]. From this work those skilled in the art will recognize that one of the utilities of the instant invention is to provide a system for in vivo as well as in vitro testing and analysis so that a correlation of HIV sequence diversity with serology of HIV neutralization, as well as other parameters can be made. The isolation and cloning of these various genes may be accomplished according to methods known to those skilled in the art. Thus this invention further provides a method for systematic identification of HIV strains and sequences for vaccine production. Incorporation of genes from primary isolates of HIV strains provides an immunogen which induces immune responses against clinical isolates of the virus and thus meets a need as yet unmet in the field. Furthermore, as the virulent isolates change, the immunogen may be modified to reflect new sequences as necessary.

To keep the terminology consistent, the following convention is followed herein for describing polynucleotide immunogen constructs:

"Vector name-HIV strain-gene-additional elements". Thus, a construct wherein the gp160 gene of the MN strain is cloned into the expression vector V1Jneo, the name it is given herein is: "V1Jneo-MN-gp160". The additional elements that are added to the construct are described in further detail below. Naturally, as the etiologic strain of the virus changes, the precise gene which is optimal for incorporation in the pharmaceutical may be changed. However, as is demonstrated below, because cytotoxic lymphocyte responses are induced which are

capable of protecting against heterologous strains, the strain variability is less critical in the immunogen and vaccines of this invention, as compared with the whole virus or subunit polypeptide based vaccines. In addition, because the pharmaceutical is easily manipulated to insert a new gene, this is an adjustment which is easily made by the standard techniques of molecular biology.

To provide a complete description of the instant invention, the following background on HIV is provided. The human immunodeficiency virus has a ribonucleic acid (RNA) genome, the structure of which is represented in Figure 1. This RNA genome must be reverse transcribed according to methods known in the art in order to produce a cDNA copy for cloning and manipulation according to the methods taught herein. At each end of the genome is a long terminal repeat which acts as a promoter. Between these termini, the genome encodes, in various reading frames, **gag-pol-env** as the major gene products: gag is the group specific antigen; pol is the reverse transcriptase, or polymerase; also encoded by this region, in an alternate reading frame, is the viral protease which is responsible for post-translational processing, for example, of gp160 into gp120 and gp41; env is the envelope protein; vif is the virion infectivity factor; REV is the regulator of virion protein expression; neg is the negative regulatory factor; vpu is the virion productivity factor "u"; tat is the trans-activator of transcription; vpr is the viral protein r. The function of each of these elements has been described (see AIDS 89, A Practical Synopsis of the V International Conference, June 4-9, 1989, Montreal, A Philadelphia Sciences Group Publication, from which figure 1 was adapted).

In one embodiment of this invention, a gene encoding an HIV or SIV protein is directly linked to a transcriptional promoter. The env gene encodes a large, membrane bound protein, gp160, which is post-translationally modified to gp41 and gp120. The gp120 gene may be placed under the control of the cytomegalovirus promoter for expression. However, gp120 is not membrane bound and therefore, upon expression, it may be secreted from the cell. As HIV tends to

remain dormant in infected cells, it is desirable that immune responses directed at cell-bound HIV epitopes also be generated. This goal is accomplished herein by expression *in vivo* of the cell-membrane associated epitope, gp160, to prime the immune system. However, expression of gp160 is repressed in the absence of REV due to non-export from the nucleus of non-spliced genes. For an understanding of this system, the life cycle of HIV must be described in further detail.

In the life cycle of HIV, upon infection of a host cell, HIV RNA genome is reverse-transcribed into a proviral DNA which integrates into host genomic DNA as a single transcriptional unit. The LTR provides the promoter which transcribes HIV genes from the 5' to 3' direction (gag, pol, env), to form an unspliced transcript of the entire genome. The unspliced transcript functions as the mRNA from which gag and pol are translated, while limited splicing must occur for translation of env encoded genes. For the regulatory gene product REV to be expressed, more than one splicing event must occur because in the genomic setting, REV and env, as is shown in figure 1, overlap. In order for transcription of env to occur, REV transcription must stop, and vice versa. In addition, the presence of REV is required for export of unspliced RNA from the nucleus. For REV to function in this manner, however, a REV responsive element (RRE) must be present on the transcript [Malim et al., *Nature* 338:254-257 (1989)].

In the polynucleotide vaccine of this invention, the obligatory splicing of certain HIV genes is eliminated by providing fully spliced genes (i.e.: the provision of a complete open reading frame for the desired gene product without the need for switches in the reading frame or elimination of noncoding regions; those of ordinary skill in the art would recognize that when splicing a particular gene, there is some latitude in the precise sequence that results; however so long as a functional coding sequence is obtained, this is acceptable). Thus, in one embodiment, the entire coding sequence for gp160 is spliced, and the sequence of REV is spliced, such that no intermittent expression of each gene product is required. Furthermore, the features of REV regulated

expression are exploited to optimize expression of HIV encoded REV-dependent, immunogenic gene products.

For REV to function as an exporter of transcripts from the nucleus to be translated in the cytoplasm, REV requires, in addition to the presence of a REV responsive element (RRE) on the transcript to be exported, at least one splice donor site on the 5' side of the gene containing the RRE [Lu et al., P.N.A.S. USA 87:7598-7602, (October 1990); Chang and Sharp, Cell 59:789-795 (December 1, 1989)]. The instant inventors conceived polynucleotides providing the REV coding sequence in a location on the same expression vector as the gene to be expressed such that co-expression of REV and the REV responsive gene occur without the need for any splicing. Thus, in a preferred embodiment of this invention, HIV genes are placed immediately downstream from a transcriptional promoter, such as the CMV promoter, and the spliced REV coding sequence is placed at a location 3' to (also referred to as downstream from) the first coding sequence. Naturally, the order of these genes could be changed. However, it may be preferable to have the immunogenic HIV cistron abut directly to the transcriptional promoter to ensure that all transcripts produced encode the entire cistron.

One method for achieving co-expression of genes relies on co-transfection of cells in culture with different vectors expressing different genes. For a REV dependent gene, the REV gene product could be provided in this manner in trans. However, this is suboptimal for the purposes of this invention, although not outside the scope of the instant invention, because of the low probability that co-transfection of a given cell would occur in vivo so as to achieve the necessary availability of REV for vigorous expression of REV dependent immunogenic HIV gene products. Another method is to provide several promoters on a given vector, each promoter controlling expression of a separate gene. This amounts to providing REV gene product in cis. This solution may be employed according to the instant invention. In such an embodiment, it would be preferable for the various promoters and the genes they control to run in opposite directions. However, because of the known

competitive interference between promoters in this type of multiple gene vector, this embodiment is also considered sub-optimal.

Ghattas et al., [Mol. and Cell. Biol. 11, No. 12:5848-5859 (Dec. 1991)], Kaufaman et al. [Nuc. Acids Res. 19, No. 16:4485-4490 (1991)], and Davies [J. Virol. 66, No. 4:1924-1932 (Apr. 1992)] have described an internal ribosome entry site (IRES) in the encephalomyocarditis virus (EMCV) leader. They reported that a system in which an upstream promoter could be used to initiate transcription of a dicistronic mRNA provides good expression of both the 5' and 3' open reading frames when an IRES is located between the two genes. Chen et al. (J. Viral., 67 : 2142-2145, 1993] have reported a system in which the 5' nontranslated region (NTR) from swine vesicular disease virus (SVDV) was used to construct a bicistronic virus for the coexpression of two genes from one transcript from an infectious viral vector.

The instant inventors have discovered that a nucleic acid construct which incorporates coordinated expression of an HIV gene containing a REV responsive element (RRE), an internal ribosome entry site (IRES) and a REV coding sequence results in efficient expression of both REV and the REV dependent gene product. This embodiment of the invention is better understood with reference to figures 2 and 3. Fig. 2 shows a generalized embodiment while, Figure 3, shows a specific embodiment of this invention which, according to the nomenclature system described above, is V1Jns-gp160(RRE)-IRES-REV. The strain of HIV from which the immunogenic HIV gene is derived is irrelevant for the illustrative purposes of this discussion, and indeed, the expression of any REV dependent gene product is predictably efficient, as is the elicitation of immune responses against both REV and the REV dependent gene product, based on the instant patent disclosure. According to the embodiment shown in Fig. 3, the vector is V1Jns, described above. Thus, the promoter (CMVintA) and terminator (BGH) are provided for by the vector, along with a prokaryotic origin of replication, to facilitate large scale production of the HIV polynucleotide vaccine through fermentation of bacteria

transformed with the construct, according to methods well known in the art. This construct does not replicate in eukaryotic tissue, due to the absence of an eukaryotic origin of replication. A splice donor site from the naturally occurring rev/tat splice donor is provided (rev/tat SD) 5 immediately preceding the HIV gene. The gag/pol/env coding sequence contains or is followed by a REV responsive element (RRE) which, upon formation of the nascent transcript, provides the necessary signals for REV binding to and export of the REV dependent mRNA from the nucleus. Next, there are sequences provided for reinitiation of 10 translation at the internal ribosome entry site (IRES) so that the downstream REV coding sequence is efficiently translated. In this manner, REV gene product is provided in cis, on the same polynucleotide as a REV dependent gene product.

In further refinements to the instant invention, a third 15 cistron may be included in the PNV. The genes encoding such immunostimulatory proteins as the B7-antigen presenting cell-surface protein, the human granulocyte/monocyte colony stimulatory factor (GM-CSF) gene, and cytokine genes such as interleukin and interferon, the use of tissue-specific transcriptional promoters and enhancers, are 20 all contemplated. The provision of B7 or GM-CSF gene in cis, either by insertion of an IRES after REV and before the B7 gene, by provision of a second promoter on the same vector construct as the dicistronic REV-dependent HIV gene, IRES-REV construct, or in trans using a 25 separate construct are all envisioned by extension of the foregoing teachings regarding REV and REV dependent genes. The generalized immuno-stimulatory effect of these gene products may be sufficient even if provided in trans to enhance immune responses against the HIV gene products encoded by the immunogen of this invention. It is preferable, particularly for B7, that the same cell presenting HIV 30 epitopes in the cleft of MHC-I molecules also present B7. This co-presentation of both the antigenic epitope and B7 "closes" the switch necessary for T-cell activation. Cytokines, particularly IL-12, which modifies whether a predominant humoral or cellular immune response is mounted [see Afonso et al., Science 263:235-237, 1994], either is

provided intravenously at the same time that PNV is introduced, or is included as a third cistron in the PNV, thereby assuring localized production of the interleukin. The genes for these immunostimulatory and immunoregulatory proteins, including GM-CSF (see Shaw and Kamen, Cell 46:659-667, 1986), interleukin-12 (see Wolf, S., et al., J. Immunol. 146:3074-3081, 1991) and B7, (see Gordon et al., J. Immunol. 143:2714-2722, 1989; for clones and sequences of newer members of the B7 family of proteins, see also Azuma, M., et al., Nature 366:76-79, 1993; and Freeman, G., et al., Science 262:909-911, 1993) are known and easily cloned and incorporated in PNV's according to this invention using methods known to the skilled practitioner. Preferably, the genes used for these purposes are the human genes so that immune responses against these proteins are minimized, allowing the expressed proteins to carry out their immunomodulatory and immunostimulatory functions. Where HIV genes have been rendered REV-independent, the REV cistron may be eliminated completely and a second cistron encoding a B7 gene family member and a third cistron encoding yet another gene-product such as IL-12, may be constructed.

The use of tissue-specific promoters or enhancers, for example the muscle creatine kinase (MCK) enhancer element, is desirable whenever it is desirable to limit expression of the polynucleotide to a particular tissue type. For example, myocytes are terminally differentiated cells which do not divide. Integration of foreign DNA into chromosomes appears to require both cell division and protein synthesis. Thus, limiting protein expression to non-dividing cells such as myocytes is preferable. However, use of the CMV promoter is adequate for achieving expression in many tissues into which the PNV is introduced.

In the various embodiments of this invention which are described below, the basic paradigm described above is used. Deviations, additions or subtractions from this basic construction design serve to hi-light the various aspects of this invention.

This patent disclosure exemplifies bi- or tri-cistronic HIV polynucleotide immunogens as polynucleotide vaccines, PNVs, to generate humoral immunity as well as cross-strain cellular antiviral immunity. The system is useful, however, for any two or three cistrons, whether or not related to HIV, when co-expression of the encoded gene products in a single cell *in vivo* is required. However, the dual humoral and cellular immune responses generated according to this invention are particularly significant to inhibiting HIV infection, given the propensity of HIV to mutate within the infected population, as well as in infected individuals. In order to formulate an effective protective vaccine for HIV it is desirable to generate both a multivalent antibody response for example to gp160 (*env* is approximately 80% conserved across various HIV-1, clade B strains, which are the prevalent strains in US human populations), the principal neutralization target on HIV, as well as cytotoxic T cells reactive to the conserved portions of gp160 and, internal viral proteins encoded by *gag*. We have made an HIV vaccine comprising gp160 genes selected from common laboratory strains; from predominant, primary viral isolates found within the infected population; from mutated gp160s designed to unmask cross-strain, neutralizing antibody epitopes; from other representative HIV genes such as the *gag* gene (>95% conserved across HIV isolates); and from SIV, which provides an animal model for testing the HIV PNV wherein non-human primates can be immunized and challenged to test viral load and progression to disease.

Virtually all HIV seropositive patients who have not advanced towards an immunodeficient state harbor anti-*gag* CTLs while about 60% of these patients show cross-strain, gp160-specific CTLs. The amount of HIV specific CTLs found in infected individuals that have progressed on to the disease state known as AIDS, however, is much lower, demonstrating the significance of our findings that we can induce cross-strain CTL responses. Because HIV late gene expression is *REV*dependent our gp160 and *gag* vaccination vectors are designed to also produce *REV* (~90% conserved), to facilitate the *REV*-dependent gene expression. An additional benefit of this invention is that anti-

REV immune responses are also generated. This gives further advantage to our vaccine because *REV* is made in large quantities very early following infection of a cell, and hours in advance of synthesis of the late gene products, thereby providing an earlier opportunity for intervention by vaccine-induced T-cell responses including CTLs and T-helper cells.

In a further embodiment of this invention, a cocktail vaccine is prepared in which different HIV REV-dependent gene constructs are mixed together to generate anti-REV CTL responses in addition to antibodies and CTL against the immunogenic HIV REV-dependent gene products. According to this embodiment, one polynucleotide encoding gp160, followed by *REV*, followed by B7, in a tri-cistronic construct having one promoter and two IRES sequences, is mixed with another polynucleotide encoding a *gag* gene product, *REV*, and B7 or another immunomodulatory or immunostimulatory gene product such as IL-12 or GM-CSF. In this fashion, with a single or several injections of polynucleotide, immune responses against several HIV related immunogens can be raised. Likewise, one polynucleotide comprising a *REV* independent gene product, such as those described in WO 93/20212, B7, and another immunomodulatory or immunostimulatory gene, such as IL-12 or GM-CSF, are mixed with another *REV*-dependent, or *REV*-independent bi- or tri-cistronic expression construct. Furthermore, multiple bi- or tri-cistronic constructs encoding HIV or other antigens could be prepared and mixed to produce a multivalent combination polynucleotide vaccine.

Immune responses induced by our *env*, *REV*, and *gag* polynucleotide vaccine constructs are demonstrated in mice, rabbits, and primates. Monitoring antibody production to *env* in mice allows confirmation that a given construct is suitably immunogenic, i.e., a high proportion of vaccinated animals show an antibody response. Mice also provide the most facile animal model suitable for testing CTL induction by our constructs and are therefore used to evaluate whether a particular construct is able to generate such activity. However, mouse cell lines have been observed to not support efficient *REV* or *tat*

functions. This observation was made in the context of HIV LTR driven expression of late genes and a limited amount of data indicates that heterologous promoters allow *REV* function in mouse cells.

5 Rabbits and monkeys (African Green, rhesus, chimpanzees) provide additional species including primates for antibody evaluation in larger, non-rodent animals. These species are also preferred to mice for antisera neutralization assays due to high levels of endogenous neutralizing activities against retroviruses observed in mouse sera.

10 These data demonstrate that sufficient immunogenicity is engendered by our vaccines to achieve protection in experiments in a chimpanzee/HIV IIIB challenge model. The currently emerging and increasingly accepted definition of protection in the scientific community is moving away from so-called "sterilizing immunity", which indicates complete protection from HIV infection, to prevention of disease. A number of correlates of this goal include reduced blood viral titer, as measured either by HIV reverse transcriptase activity, by infectivity of samples of serum, by ELISA assay of p24 or other HIV antigen concentration in blood, increased CD4⁺ T-cell concentration, 15 and by extended survival rates [see, for example, Cohen, J., Science 262:1820-1821, 1993, for a discussion of the evolving definition of anti-HIV vaccine efficacy]. The immunogens of the instant invention also generate neutralizing immune responses against infectious (clinical, primary field) isolates of HIV.

20

25 **Immunology**

A. Antibody Responses to env.

30 1. gp160 and gp120. An ELISA assay is used to determine whether vaccine vectors expressing either secreted gp120 or membrane-bound gp160 are efficacious for production of *env*-specific antibodies. Initial in vitro characterization of *env* expression by our vaccination vectors is provided by immunoblot analysis of gp160 transfected cell lysates. These data confirm and quantitate gp160 expression using anti-gp41 and anti-gp120 monoclonal antibodies to visualize transfectant cell gp160 expression. In one embodiment of this invention, gp160 is

preferred to gp120 for the following reasons: (1) an initial gp120 vector gave inconsistent immunogenicity in mice and was very poorly or non-responsive in African Green Monkeys; (2) gp160 contributes additional neutralizing antibody as well as CTL epitopes by providing the addition of approximately 190 amino acid residues due to the inclusion of gp41; (3) gp160 expression is more similar to viral *env* with respect to tetramer assembly and overall conformation; and (4) we find that, like the success of membrane-bound, influenza HA constructs for producing neutralizing antibody responses in mice, ferrets, and nonhuman primates [see Ulmer et al., Science 259:1745-1749, 1993; Montgomery, D., et al., DNA and Cell Biol., 12:777-783, 1993] anti-gp160 antibody generation is superior to anti-gp120 antibody generation. Selection of which type of *env*, or whether a cocktail of *env* subfragments, is preferred is determined by the experiments outlined below.

2. Presence and Breadth of Neutralizing Activity. ELISA positive antisera from rabbits and monkeys is tested and shown to neutralize both homologous and heterologous HIV strains.

3. V3 vs. non-V3 Neutralizing Antibodies. A major goal for *env* PNVs is to generate broadly neutralizing antibodies. It has now been shown that antibodies directed against V3 loops are very strain specific, and the serology of this response has been used to define strains.

a. Non-V3 neutralizing antibodies appear to primarily recognize discontinuous, structural epitopes within gp120 which are responsible for CD4 binding. Antibodies to this domain are polyclonal and more broadly cross-neutralizing probably due to restraints on mutations imposed by the need for the virus to bind its cellular ligand. An in vitro assay is used to test for blocking gp120 binding to CD4 immobilized on 96 well plates by sera from immunized animals. A second in vitro assay detects direct antibody binding to synthetic peptides representing selected V3 domains immobilized on plastic.

These assays are compatible for antisera from any of the animal types used in our studies and define the types of neutralizing antibodies our vaccines have generated as well as provide an in vitro correlate to virus neutralization.

5 b. gp41 harbors at least one major neutralization determinant, corresponding to the highly conserved linear epitope recognized by the broadly neutralizing 2F5 monoclonal antibody (commercially available from Viral Testing Systems Corp., Texas Commerce Tower, 600 Travis Street, Suite 4750, Houston, TX 77002-3005(USA), or Waldheim Pharmazeutika GmbH, Boltzmangasse 11, A-1091 Wien, Austria), as well as other potential sites including the well-conserved "fusion peptide" domain located at the N-terminus of gp41. Besides the detection of antibodies directed against gp41 by immunoblot as described above, an in vitro assay test is used for antibodies which bind to synthetic peptides representing these domains immobilized on plastic.

20 4. Maturation of the Antibody Response. In HIV seropositive patients, the neutralizing antibody responses progress from chiefly anti-V3 to include more broadly neutralizing antibodies comprising the structural gp120 domain epitopes described above (#3), including gp41 epitopes. These types of antibody responses are monitored over the course of both time and subsequent vaccinations.

25 B. T Cell Reactivities Against *env*, *REV*, *nef* and *gag*.

30 1. Generation of CTL Responses. Viral proteins which are synthesized within cells give rise to MHC I-restricted CTL responses. Each of these proteins elicit CTL in seropositive patients. Our vaccines also are able to elicit CTL in mice. The immunogenetics of mouse strains are conducive to such studies, as demonstrated with influenza NP, [see Ulmer et al., Science 259:1745-1749, 1993]. Several epitopes have been defined for the HIV proteins env, REV, *nef* and *gag* in Balb/c mice, thus facilitating in vitro CTL culture and cytotoxicity assays. Additionally, it is advantageous to use syngenic tumor lines,

such as the murine mastocytoma P815, transfected with these genes to provide targets for CTL as well as for in vitro antigen specific restimulation. Methods for defining immunogens capable of eliciting MHC class I-restricted cytotoxic T lymphocytes are known [see Calin-Laurens, et al., Vaccine 11(9):974-978, 1993; see particularly Eriksson, et al., Vaccine 11(8):859-865, 1993, wherein T-cell activating epitopes on the HIV gp120 were mapped in primates and several regions, including gp120 amino acids 142-192, 296-343, 367-400, and 410-453 were each found to induce lymphoproliferation; furthermore, discrete regions 248-269 and 270-295 were lymphoproliferative. A peptide encompassing amino acids 152-176 was also found to induce HIV neutralizing antibodies], and these methods may be used to identify immunogenic epitopes for inclusion in the PNV of this invention.

Alternatively, the entire gene encoding gp160, gp120, protease, or gag could be used. For additional review on this subject, see for example, Shirai et al., J. Immunol. 148:1657-1667, 1992; Choppin et al., J. Immunol. 147:569-574, 1991; Choppin et al., J. Immunol. 147:575-583, 1991; Berzofsky et al., J. Clin. Invest. 88:876-884, 1991. As used herein, T-cell effector function is associated with mature T-cell phenotype, for example, cytotoxicity, cytokine secretion for B-cell activation, and/or recruitment or stimulation of macrophages and neutrophils.

2. Measurement of TH Activities. Spleen cell cultures derived from vaccinated animals are tested for recall to specific antigens by addition of either recombinant protein or peptide epitopes. Activation of T cells by such antigens, presented by accompanying splenic antigen presenting cells, APCs, is monitored by proliferation of these cultures or by cytokine production. The pattern of cytokine production also allows classification of TH response as type 1 or type 2. Because dominant TH2 responses appear to correlate with the exclusion of cellular immunity in immunocompromised seropositive patients, it is possible to define the type of response engendered by a given PNV in patients, permitting manipulation of the resulting immune responses.

5 3. Delayed Type Hypersensitivity (DTH). DTH to viral antigen after i.d. injection is indicative of cellular, primarily MHC II-restricted, immunity. Because of the commercial availability of recombinant HIV proteins and synthetic peptides for known epitopes, DTH responses are easily determined in vaccinated vertebrates using these reagents, thus providing an additional in vivo correlate for inducing cellular immunity.

10 Protection

Based upon the above immunologic studies, it is predictable that our vaccines are effective in vertebrates against challenge by virulent HIV. These studies are accomplished in an HIVIIIB/chimpanzee challenge model after sufficient vaccination of these animals with a PNV construct, or a cocktail of PNV constructs comprised of gp160IIIB, gagIIIB, nefIIIB and REVIIIB. The IIIB strain is useful in this regard as the chimpanzee titer of lethal doses of this strain has been established. However, the same studies are envisioned using any strain of HIV and the epitopes specific to or heterologous to the given strain. A second vaccination/challenge model, in addition to chimpanzees, is the scid-hu PBL mouse. This model allows testing of the human lymphocyte immune system and our vaccine with subsequent HIV challenge in a mouse host. This system is advantageous as it is easily adapted to use with any HIV strain and it provides evidence of protection against multiple strains of primary field isolates of HIV. A third challenge model utilizes hybrid HIV/SIV viruses (SHIV), some of which have been shown to infect rhesus monkeys and lead to immunodeficiency disease resulting in death [see Li, J., et al., J. AIDS 5:639-646, 1992]. Vaccination of rhesus with our polynucleotide vaccine constructs is protective against subsequent challenge with lethal doses of SHIV.

PNV Construct Summary

HIV and other genes are preferably ligated into an expression vector which has been specifically optimized for polynucleotide vaccinations. According to this invention disclosure methods for producing several such vectors are enabled. Essentially all extraneous DNA is removed, leaving the essential elements of transcriptional promoter, immunogenic epitopes, and additional cistrons encoding immunoenhancing or immunomodulatory genes, with their own promoters or IRES, transcriptional terminator, bacterial origin of replication and antibiotic resistance gene, as previously described (see figure 2). Those skilled in the art will appreciate that introduction of RNA which has been transcribed in vitro to produce the multi-cistronic mRNAs encoded by the DNA counterparts of this invention naturally forms an integral part of this invention. For this purpose, it is desirable to use as the transcriptional promoter such powerful RNA polymerase promoters as the T7 or SP6 promoters, and performing run-on transcription with a linearized DNA template. These methods are well known in the art.

Expression of HIV late genes such as *env* and *gag* is *REV*-dependent and requires that the *REV* response element (RRE) be present on the viral gene transcript. A secreted form of gp120 can be generated in the absence of *REV* by substitution of the gp120 leader peptide with a heterologous leader such as from tPA (tissue-type plasminogen activator), and preferably by a leader peptide such as is found in highly expressed mammalian proteins such as immunoglobulin leader peptides. We have inserted a tPA-gp120 chimeric gene into V1Jns which efficiently expresses secreted gp120 in transfected cells (RD, a human rhabdomyosarcoma line). We have also developed an IRES-based (IRES = internal ribosomal entry site) dicistronic V1Jns vector containing both gp160 (which harbors the RRE) and *REV* which efficiently expresses gp160 in transfected cell lines (293, a human embryonic kidney cell line; and RD). Monocistronic gp160 does not produce any protein upon transfection without the addition of a *REV* expression vector. Dicistronic gp160/*REV* produces similar amounts of gp160 as co-transfected gp160 and *REV* monocistronic vectors.

From these studies, it is predictable that dicistronic vectors more efficiently express gp160 following introduction *in vivo* intramuscularly relative to a mixture of gp160 and *REV* vectors because the dicistron insures the proximity of gp160 construct and *REV* within structurally extended, multi-nucleated muscle cells. This dicistronic strategy also supports expression of *gag* after the inclusion of the RRE within the transcript region of the vector. It also supports the expression of unrelated genes in a bi- or tri-cistronic PNV, such as co-expression of HIV immunogenic epitopes, influenza virus immunogenic epitopes, cancer-related antigens, and immunomodulatory genes such as interleukin, B7 and GM-CSF.

15 Representative Construct Components Include (but are not restricted to)
(see figure 2, Cistrons I, II, and III):

1. tPA-gp120MN;
2. gp160IIIB/IRES/REVIIIB;
3. gp160IIIB;
4. REVIIIB;
5. *tat/REV/gp160* (a genomic IIIB clone which weakly expresses gp160);
6. REV/gp160;
7. gp160MN;
8. gp160 from clinically relevant primary HIV isolates;
9. nef, using the gene from clinically relevant strains;
10. *gag*IIIB: for anti-*gag* CTL;
11. tPA-gp120IIIB: for chimp studies;
12. gp160 with structural mutations: V3 loop substitutions from clinically relevant strains of HIV; several mutations on several constructs such as variable loop removal, Asn mutations to remove steric carbohydrate obstacles to structural, neutralizing antibody epitopes; and CD4 binding site knockout mutants;
- 30 13. gp41: to specifically elicit anti-gp41 neutralizing

antibodies, particularly the 2F5 monoclonal antibody epitope, located directly anterior to the transmembrane domain, which is broadly conserved across many strains.

5 This peptide is difficult to express in the absence of gp120 and requires several strategies, e.g., a recent report found that the 2F5 epitope spliced into an influenza HA loop tip could elicit HIV neutralizing antibodies; alternatively, provision of appropriate leader sequences, as in the tPA signal peptide leader sequence, allows expression of this

10 gene product;

14. *gag*: similar to construct from #5 above, using the gene from clinically relevant strains;

15 15. *rev*: for gp160 and *gag* dicistronics;

16. B7 coding sequences;

17. GM-CSF sequences;

18. Interleukin sequences, particularly encoding IL-12;

19. Tumor associated antigens;

20. Genes encoding antigens expressed by pathogens other than HIV, such as, but not limited to, influenza virus nucleoprotein, hemagglutinin, matrix, neuraminidase, and other antigenic proteins; herpes simplex virus genes; human papillomavirus genes; tuberculosis antigens; hepatitis A, B, or C virus antigens; and combinations of these and other antigens to form at least dicistronic constructs which may be combined with multiple other polycistronic constructs to provide a cocktail composition capable of raising immune responses against all of the represented pathogens or tumor antigens.

25

30 In the HIV env constructs, those of ordinary skill in the art will recognize the desirability of expressing nucleic acids encoding various env V3 loop amino acid sequences. As an example, any or all of the following amino acid sequences, or portions thereof, may be encoded by HIV polynucleotide immunogens of this invention:

- 33 -

**GP160 V3 LOOP SEQUENCE SUMMARY FOR PNV
CONSTRUCTS**

- 5 North American/European Consensus, SEQ.ID:1:
CysThrArgProAsnAsnAsnThrArgLysSerIleHisIle**GlyProGlyArgAla**
PheTyrThrThrGlyGluIleIle**GlyAspIleArgGlnAlaHisCys**
- 10 MN, SEQ.ID:2:
CysThrArgProAsnTyrAsnLysArgLysArgIleHisIle**GlyProGlyArgAla**
PheTyrThrThrLysAsnIleIle**GlyThrIleArgGlnAlaHisCys**
- 15 IIIB (HXB2R), SEQ.ID:3:
CysThrArgProAsnAsnAsnThrArgLysArgIleArgIle**GlnArgGlyProGly**
ArgAlaPheValThrIle**GlyLysIleGlyAsnMetArgGlnAlaHisCys**
- 20 116-v, SEQ.ID:4:
CysThrArgProAsnAsnAsnThrArgLys**GlyIleHisIleGlyProGlyArgAla**
PheTyrThrThrGlyLysIleIle**GlyAsnIleArgGlnAlaHisCys**
- 25 452-p, SEQ.ID:5:
CysThrArgProSerAsnAsnAsnThrArgLysSerIleHisIle**GlyProGlyLys**
AlaPheTyrAlaThrGlyAlaIleIle**GlyAspIleArgGlnAlaHisCys**
- 146-v, SEQ.ID:6:
25 CysThrArgProAsnAsnAsnThrArgArgSerIleHisIle**AlaProGlyArgAla**
PheTyrAlaThrGly**AspIleIleGlyAspIleArgGlnAlaHisCys**

30 The protective efficacy of polynucleotide HIV immunogens against subsequent viral challenge is demonstrated by immunization with the non-replicating plasmid DNA of this invention. This is advantageous since no infectious agent is involved, no assembly of virus particles is required, and determinant selection is permitted. Furthermore, because the sequence of *gag* and protease and several of the other viral gene products is conserved among various strains of

HIV, protection against subsequent challenge by a virulent strain of HIV that is homologous to, as well as strains heterologous to the strain from which the cloned gene is obtained, is enabled.

5 The i.m. injection of a DNA expression vector encoding gp160 results in the generation of significant protective immunity against subsequent viral challenge. In particular, gp160-specific antibodies and primary CTLs are produced. Immune responses directed against conserved proteins can be effective despite the antigenic shift and drift of the variable envelope proteins. Because each of the HIV 10 gene products exhibit some degree of conservation, and because CTLs are generated in response to intracellular expression and MHC processing, it is predictable that many virus genes give rise to responses analogous to that achieved for gp160. Thus, many of these genes have been cloned, as shown by the cloned and sequenced junctions in the 15 expression vector (see below) such that these constructs are immunogenic agents in available form.

The invention offers a means to induce cross-strain protective immunity without the need for self-replicating agents or adjuvants. In addition, immunization with the instant polynucleotides 20 offers a number of other advantages. First, this approach to vaccination should be applicable to tumors as well as infectious agents, since the CD8⁺ CTL response is important for both pathophysiological processes [K. Tanaka *et al.*, Annu. Rev. Immunol. 6, 359 (1988)]. Therefore, eliciting an immune response against a protein crucial to the 25 transformation process may be an effective means of cancer protection or immunotherapy. Second, the generation of high titer antibodies against expressed proteins after injection of viral protein and human growth hormone DNA, [see for example D.-c. Tang *et al.*, Nature 356, 152, 1992], indicates this is a facile and highly effective means of 30 making antibody-based vaccines, either separately or in combination with cytotoxic T-lymphocyte vaccines targeted towards conserved antigens.

The ease of producing and purifying DNA constructs compares favorably with traditional protein purification, facilitating the

generation of combination vaccines. Thus, multiple constructs, for example encoding gp160, gp120, gp41, or any other HIV gene may be prepared, mixed and co-administered. Finally, because protein expression is maintained following DNA injection [H. Lin *et al.*, Circulation **82**, 2217 (1990); R.N. Kitsis *et al.*, Proc. Natl. Acad. Sci. (USA) **88**, 4138 (1991); E. Hansen *et al.*, FEBS Lett. **290**, 73 (1991); S. Jiao *et al.*, Hum. Gene Therapy **3**, 21 (1992); J.A. Wolff *et al.*, Human Mol. Genet. **1**, 363 (1992)], the persistence of B- and T-cell memory may be enhanced [D. Gray and P. Matzinger, J. Exp. Med. **174**, 969 (1991); S. Oehen *et al.*, *ibid.* **176**, 1273 (1992)], thereby engendering long-lived humoral and cell-mediated immunity.

The standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of the DNA immunogens of this invention. While standard techniques of molecular biology are therefore sufficient for the production of the products of this invention, the specific constructs disclosed herein provide polynucleotide immunogens which surprisingly produce cross-strain and primary HIV isolate neutralization, a result heretofore unattainable with standard inactivated whole virus or subunit protein vaccines.

The amount of expressible DNA or transcribed RNA to be introduced into a vaccine recipient will depend on the strength of the transcriptional and translational promoters used and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 1 ng to 100 mg, and preferably about 10 µg to 300 µg is administered directly into muscle tissue. Subcutaneous injection, intradermal introduction, impression through the skin, and other modes of administration such as intraperitoneal, intravenous, or inhalation delivery are also contemplated. It is also contemplated that booster vaccinations are to be provided. Following vaccination with HIV polynucleotide immunogen, boosting with HIV protein immunogens such as gp160, gp120, and gag gene products is also contemplated. Parenteral administration, such as intravenous, intramuscular, subcutaneous or other means of

administration of interleukin-12 protein, concurrently with or subsequent to parenteral introduction of the PNV of this invention are advantageous.

The polynucleotide may be naked, that is, unassociated with any proteins, adjuvants or other agents which impact on the recipient's immune system. In this case, it is desirable for the polynucleotide to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline. Alternatively, the DNA may be associated with liposomes, such as lecithin liposomes or other liposomes known in the art, as a DNA-liposome mixture, or the DNA may be associated with an adjuvant known in the art to boost immune responses, such as a protein or other carrier. Agents which assist in the cellular uptake of DNA, such as, but not limited to, calcium ions, may also be used to advantage. These agents are generally referred to herein as transfection facilitating reagents and pharmaceutically acceptable carriers. Techniques for coating microprojectiles coated with polynucleotide are known in the art and are also useful in connection with this invention.

Accordingly, one embodiment of this invention is a polynucleotide which, upon introduction into mammalian tissue, induces the co-expression in a single cell, in vivo, of two or three different discrete gene products, comprising:

a first transcriptional promoter which operates efficiently in eukaryotic cells upstream from and in transcriptional control of a first cistron;

a second cistron downstream from the first cistron, under transcriptional control either of the first transcriptional promoter, or under control of a second transcriptional promoter;

optionally, a third cistron downstream from the second cistron, under transcriptional control either of the first transcriptional promoter, or under control of a second transcriptional promoter, or under control of a third transcriptional promoter;

a transcriptional terminator following each of the first, second and third cistron, unless followed by another cistron lacking its own transcriptional promoter.

In another embodiment, the invention is a polynucleotide which comprises contiguous nucleic acid sequences which cannot replicate in eukaryotic cells but which are capable of being expressed to produce a gene product upon introduction of the polynucleotide into eukaryotic tissues in vivo. The encoded gene product preferably either acts as an immunostimulant or as an antigen capable of generating an immune response. Thus, the nucleic acid sequences in this embodiment encode a spliced REV gene, a human immunodeficiency virus (HIV) immunogenic epitope, and optionally, a cytokine or a T-cell costimulatory element, such as a member of the B7 family of proteins.

In another embodiment, the invention is a method for co-expression in a single cell, in vivo, of two or three different, discrete gene products, which comprises introducing between about 0.1 µg and 100 mg of a polynucleotide of this invention into the tissue of the vertebrate.

In another embodiment, the invention is a method for using a REV dependent HIV gene to induce immune responses in vivo which comprises:

- 20 a) isolating the REV dependent HIV gene;
- b) linking the isolated gene to regulatory sequences such that the gene is expressible by virtue of being operatively linked to control sequences which, when introduced into a living tissue, direct the transcription initiation and subsequent translation of the gene;
- 25 c) introducing the expressible gene into a living tissue;
- d) introducing a gene encoding HIV REV either in trans or in cis to the HIV REV dependent gene; and
- e) optionally, boosting with additional expressible HIV gene.

30 A further embodiment of this invention amounts to a method of inducing an antigen presenting cell to stimulate cytotoxic T-cell proliferation specific to HIV antigens. This involves exposing cells of a vertebrate in vivo to a polynucleotide which consists of an antigenic HIV epitope, REV if the antigenic HIV epitope depends on REV for efficient expression, and B7 encoding sequences.

- 36 -

The following examples are provided to further define the invention, without limiting the invention to the specifics of the examples.

5 Materials descriptions

Vectors pF411 and pF412: These vectors were subcloned from vector pSP62 which was constructed in R. Gallo's lab. pSP62 is an available reagent from Biotech Research Laboratories, Inc. pSP62 has a 12.5 kb XbaI fragment of the HXB2 genome subcloned from lambda HXB2. Sall and Xba I digestion of pSP62 yields to HXB2 fragments: 5'-XbaI/Sall, 6.5 kb and 3'- Sall/XbaI, 6 kb. These inserts were subcloned into pUC 18 at SmaI and Sall sites yielding pF411 (5'-XbaI/Sall) and pF412 (3'-XbaI/Sall). pF411 contains gag/pol and pF412 contains tat/rev/env/nef.

10
15
20
25
Repligen reagents:
recombinant rev (IIIB), #RP1024-10
rec. gp120 (IIIB), #RP1001-10
anti-rev monoclonal antibody, #RP1029-10
anti-gp120 mAB, #1C1, #RP1010-10

AIDS Research and Reference Reagent Program:
anti-gp41 mAB hybridoma, Chessie 8, #526

EXAMPLE 1
VECTORS FOR VACCINE PRODUCTION

A) V1: The expression vector V1 was constructed from pCMVIE-AKI-DHFR [Y. Whang *et al.*, J. Virol. **61**, 1796 (1987)]. The AKI and DHFR genes were removed by cutting the vector with EcoR I and self-ligating. This vector does not contain intron A in the CMV promoter, so it was added as a PCR fragment that had a deleted internal Sac I site [at 1855 as numbered in B.S. Chapman *et al.*, Nuc. Acids Res. **19**, 3979 (1991)]. The template used for the PCR reactions was pCMVintA-Lux, made by ligating the Hind III and Nhe I fragment from pCMV6a120

[see B.S. Chapman *et al.*, *ibid.*,] which includes hCMV-IE1 enhancer/promoter and intron A, into the Hind III and Xba I sites of pBL3 to generate pCMVIntBL. The 1881 base pair luciferase gene fragment (Hind III-Sma I Klenow filled-in) from RSV-Lux [J.R. de Wet *et al.*, Mol. Cell Biol. 7, 725, 1987] was cloned into the Sal I site of pCMVIntBL, which was Klenow filled-in and phosphatase treated.

The primers that spanned intron A are:
5' primer, SEQ. ID:7:
10 5'-CTATATAAGCAGAG CTCGTTAG-3'; The 3' primer, SEQ ID:8:
5'-TAGCAAAGATCTAAGGACGGTGA CTGCAG-3'.

The primers used to remove the Sac I site are:
sense primer, SEQ ID:9:
15 5-GTATGTGTCTGAAAATGAGCGTGGAGATTGGGCTCGCAC-3'
and the antisense primer, SEQ ID:10,:
5'-GTGCGAGCCCAATCTCCACGGCTCATTTCAGACACA TAC-3'.

20 The PCR fragment was cut with Sac I and Bgl II and inserted into the vector which had been cut with the same enzymes.

B) V1J EXPRESSION VECTOR, SEQ. ID:12:

Our purpose in creating V1J was to remove the promoter
25 and transcription termination elements from our vector, V1, in order to place them within a more defined context, create a more compact vector, and to improve plasmid purification yields.

V1J is derived from vectors V1, (see Example 1) and pUC18, a commercially available plasmid. V1 was digested with SspI
30 and EcoRI restriction enzymes producing two fragments of DNA. The smaller of these fragments, containing the CMVintA promoter and Bovine Growth Hormone (BGH) transcription termination elements which control the expression of heterologous genes (SEQ ID:13:), was purified from an agarose electrophoresis gel. The ends of this DNA

- 40 -

fragment were then "blunted" using the T4 DNA polymerase enzyme in order to facilitate its ligation to another "blunt-ended" DNA fragment.

pUC18 was chosen to provide the "backbone" of the expression vector. It is known to produce high yields of plasmid, is well-characterized by sequence and function, and is of minimum size. We removed the entire *lac* operon from this vector, which was unnecessary for our purposes and may be detrimental to plasmid yields and heterologous gene expression, by partial digestion with the HaeII restriction enzyme. The remaining plasmid was purified from an agarose electrophoresis gel, blunt-ended with the T4 DNA polymerase, treated with calf intestinal alkaline phosphatase, and ligated to the CMVintA/BGH element described above. Plasmids exhibiting either of two possible orientations of the promoter elements within the pUC backbone were obtained. One of these plasmids gave much higher yields of DNA in *E. coli* and was designated V1J (SEQ. ID:12:). This vector's structure was verified by sequence analysis of the junction regions and was subsequently demonstrated to give comparable or higher expression of heterologous genes compared with V1.

20 C) V1Jneo EXPRESSION VECTOR, SEQ. ID:14:

It was necessary to remove the *amp^r* gene used for antibiotic selection of bacteria harboring V1J because ampicillin may not be used in large-scale fermenters. The *amp^r* gene from the pUC backbone of V1J was removed by digestion with SspI and Eam1105I restriction enzymes. The remaining plasmid was purified by agarose gel electrophoresis, blunt-ended with T4 DNA polymerase, and then treated with calf intestinal alkaline phosphatase. The commercially available *kan^r* gene, derived from transposon 903 and contained within the pUC4K plasmid, was excised using the *Pst*I restriction enzyme, purified by agarose gel electrophoresis, and blunt-ended with T4 DNA polymerase. This fragment was ligated with the V1J backbone and plasmids with the *kan^r* gene in either orientation were derived which were designated as V1Jneo #'s 1 and 3. Each of these plasmids was confirmed by restriction enzyme digestion analysis, DNA sequencing of

the junction regions, and was shown to produce similar quantities of plasmid as V1J. Expression of heterologous gene products was also comparable to V1J for these V1Jneo vectors. We arbitrarily selected 5 V1Jneo#3, referred to as V1Jneo hereafter (SEQ. ID:14:), which contains the kan^r gene in the same orientation as the amp^r gene in V1J as the expression construct.

D) V1Jns EXPRESSION VECTOR:

An Sfi I site was added to V1Jneo to facilitate integration 10 studies. A commercially available 13 base pair Sfi I linker (New England BioLabs) was added at the Kpn I site within the BGH sequence of the vector. V1Jneo was linearized with Kpn I, gel purified, blunted by T4 DNA polymerase, and ligated to the blunt Sfi I linker. Clonal isolates were chosen by restriction mapping and verified by sequencing 15 through the linker. The new vector was designated V1Jns. Expression of heterologous genes in V1Jns (with Sfi I) was comparable to expression of the same genes in V1Jneo (with Kpn I).

E) pGEM-3-IRES: The encephalomyocarditis virus (EMCV) internal 20 ribosomal entry site (IRES) allows efficient expression of two genes within a single mRNA transcript when it is juxtaposed between them. We have utilized this non-coding gene segment to create dicistronic 25 expression vectors for polynucleotide vaccines. The EMCV IRES segment was subcloned as a 0.6 kb EcoR1/BssHII digestion fragment from the pCITE-1 plasmid (Novagen). This fragment was agarose gel-purified, blunt-ended using T4 DNA polymerase and subsequently 30 ligated into pGEM-3 (Promega) which had been XbaI-digested, blunt-ended with T4 DNA polymerase, and phosphatased. Clones were obtained for each of the two possible orientations of this DNA within pGEM-3 and each junction site verified by DNA sequencing. The preferred orientation for subsequent construction of dicistronic vectors positioned the NcoI site within the IRES proximal to BamHI site within pGEM-3. This vector is referred to as pGEM-3-IRES.

5 F) pGEM-3-IRES*: A second IRES vector was prepared containing mutations in the IRES sequence (IRES*) conferred by a PCR oligomer which may optimize IRES-driven expression compared to wild type IRES. PCR amplification of IRES* was performed using pCITE-1 plasmid (Novagen) with the following sense and antisense oligomers:
10 5'-GGT ACA AGA TCT ACT ATA GGG AGA CCG GAA TTC CGC-3', SEQ. ID:11:, and 5'-CCA CAT AGA TCT GTT CCA TGG TTG TGG CAA TAT TAT CAT CG-3', SEQ. ID:15:, respectively. The mutated residue, underlined in the antisense codon, eliminates an upstream ATG from the preferred ATG contained within the NcoI/Kozak sequence at the 3'-terminal end of the IRES

15 G) pGEM-3-IRES/REV: HIVIIb *REV* was PCR amplified from pCV-1 (catalogue #303, NIH AIDS Research and Reference Program) using synthetic oligomers. The sense and antisense oligomers were 5'-GGT ACA AGA TCT ACC ATG GCA GGA AGA AGC GGA GAC AGC-3', SEQ. ID:16:, and 5'-CCA CAT AGA TCT GAT ATC GCA CTA TTC TTT AGC TCC TGA CTC C-3', SEQ. ID:17:, respectively.
20 These oligomers provide BglII sites at either end of the translation open reading frame as well as an EcoRV site directly upstream from the BglII site at the 3'-terminal end of *rev*. After PCR, the *REV* gene was treated with NcoI (located within the Kozak sequence) and BglII restriction enzymes and ligated with pGEM-3-IRES which had been treated with NcoI and BamHI restriction enzymes. Each ligation junction as well as the entire 0.3 kb *REV* gene was confirmed by DNA sequencing.
25

30 H) V1Jns-tPA: In order to provide an heterologous leader peptide sequence to secreted and/or membrane proteins, V1Jn was modified to include the human tissue-specific plasminogen activator (tPA) leader. Two synthetic complementary oligomers were annealed and then ligated into V1Jn which had been BglII digested. The sense and antisense oligomers were 5'-GATC ACC ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG CTG TGT GGA GCA GTC TTC GTT

TCG CCC AGC GA-3', SEQ.ID:18:, and 5'-GAT CTC GCT GGG TGA
AAC GAA GAC TGC TCC ACA CAG CAG CAC ACA GC:
GAG CCC TCT CTT CAT TGC ATC CAT GGT-3', SEQ. ID:19:. The
Kozak sequence is underlined in the sense oligomer. These oligomers
5 have overhanging bases compatible for ligation to BglII-cleaved
sequences. After ligation the upstream BglII site is destroyed while the
downstream BglII is retained for subsequent ligations. Both the
junction sites as well as the entire tPA leader sequence were verified by
10 DNA sequencing. Additionally, in order to conform with our consensus
optimized vector V1Jns (=V1Jneo with an SfiI site), an SfiI restriction
site was placed at the KpnI site within the BGH terminator region of
V1Jn-tPA by blunting the KpnI site with T4 DNA polymerase followed
by ligation with an SfiI linker (catalogue #1138, New England BioLabs).
15 This modification was verified by restriction digestion and agarose gel
electrophoresis.

I) V1Jns-HIVIIIb REV: *REV* was amplified by PCR as described
above for pGEM-3-IRES/*REV*, digested with BglII restriction enzyme,
20 and ligated into V1Jns which had been BglII- and calf intestinal alkaline
phosphatase-treated. Ligation junctions were confirmed by DNA
sequencing and expression of *REV* was verified by in vitro transfection
of RD cells and immunoblot analysis (greater than 1 µg *REV* obtained
per 10⁶ cells).

25 J) pGEM-3-RRE/IRES/REV: In order to make a cassette consisting of
the *REV* response element (RRE) which is required to be on an RNA
transcript in order for *REV*-dependent expression to occur, the RRE
from HIV strain HXB2 was obtained by PCR using the following
30 synthetic oligomers: sense oligomer, 5'-GGT ACA TGA TCA GA-
ATC GCCC GGG C CGA GAT CTT CAG ACT TGG AGG AGG -
3', SEQ.ID:20:; and antisense oligomer, 5'-CCA CAT TGA TCA C
CTT GTG TAA TTG TTA ATT TCT CTG TCC-3', SEQ.ID:21:.
These oligomers provide BclI restriction sites at either end of the insert
as well as EcoRV and SrfI sites at the 5'-end of the insert. The RRE

was blunt-end ligated into pGEM-3-/IRES/REV at the HincII restriction site which precedes IRES. The ligation products were verified by restriction enzyme mapping and by DNA sequencing across the ligation junctions.

5

EXAMPLE 2

gp120 Vaccines:

Expression of the *REV* -dependent *env* gene as gp120 was conducted as follows: gp120 was PCR-cloned from the MN strain of HIV with either the native leader peptide sequence (V1Jns-gp120), or as a fusion with the tissue-plasminogen activator (tPA) leader peptide replacing the native leader peptide (V1Jns-tPA-gp120). tPA-gp120 expression has been shown to be *REV*-independent [B.S. Chapman *et al.*, Nuc. Acids Res. **19**, 3979 (1991); it should be noted that other leader sequences would provide a similar function in rendering the gp120 gene *REV* independent]. This was accomplished by preparing the following gp120 constructs utilizing the above described vectors:

20

I. gp120 VACCINE CONSTRUCTS:

A) V1Jns-tPA-HIVMN gp120: HIVMN gp120 gene (Medimmune) was PCR amplified using oligomers designed to remove the first 30 amino acids of the peptide leader sequence and to facilitate cloning into V1Jns-tPA creating a chimeric protein consisting of the tPA leader peptide followed by the remaining gp120 sequence following amino acid residue 30. This design allows for *REV* -independent gp120 expression and secretion of soluble gp120 from cells harboring this plasmid. The sense and antisense PCR oligomers used were 5'-CCC CGG ATC CTG ATC ACA GAA AAA TTG TGGGTC ACA GTC-3', SEQ. ID:22:, and 5'-C CCC AGG AATC CAC CTG TTA GCG CTT TTC TCT CTG CAC CAC TCT TCT C-3', SEQ. ID:23:. The translation stop codon is underlined. These oligomers contain BamHI restriction enzyme sites at either end of the translation open reading frame with a BclI site located 3' to the BamHI of the sense oligomer. The PCR product was sequentially digested with BclI followed by BamHI and ligated into

V1Jns-tPA which had been BglII digested followed by calf intestinal alkaline phosphatase treatment. The resulting vector was sequenced to confirm inframe fusion between the tPA leader and gp120 coding sequence, and gp120 expression and secretion was verified by immunoblot analysis of transfected RB cells. Thus, this vector encoding the tPA-HIVMN-gp120 is useful for inclusion in a bi- or tri-cistronic construct expressing gag, B7 or other antigens.

B) V1-tPA-HIV MN gp120: A slightly different version of the chimeric tPA-HIV MN gp120 vector described above was made using an earlier version of our basic vaccine expression vector, V1 (see Nucleic Acid Pharmaceuticals patent), which contained a somewhat different tPA peptide leader sequence from that described for V1Jns-tPA.

In either of the foregoing PNV constructs, provision of an IRES sequence after the translation stop codon, and downstream cloning of immunomodulatory genes such as B7, provides bi- or tri-cistronic polynucleotides useful according to the method of this invention. These PNV's efficiently express both gene products.

C) V1Jns-tPA-HIV IIIB gp120: This vector is analogous to I.A. except that the HIV IIIB strain was used for gp120 sequence. The sense and antisense PCR oligomers used were: 5'-GGT ACA TGA TCA CA GAA AAA TTG TGG GTC ACA GTC-3', SEQ.ID:24:, and 5'-CCA CAT TGA TCA GAT ATC TTA TCT TTT TTC TCT CTG CAC CAC TCT TC-3', SEQ.ID:25:, respectively. These oligomers provide BclI sites at either end of the insert as well as an EcoRV just upstream of the BclI site at the 3'-end. The 5'-terminal BclI site allows ligation into the BglII site of V1Jns-tPA to create a chimeric tPA-gp120 gene encoding the tPA leader sequence and gp120 without its native leader sequence. Ligation products were verified by restriction digestion and DNA sequencing.

II. IN VITRO gp120 VACCINE EXPRESSION:

5 In vitro expression was tested in transfected human rhabdomyosarcoma (RD) cells for these constructs. Quantitation of secreted tPA-gp120 from transfected RD cells showed that V1Jns-tPA-gp120 vector produced secreted gp120.

III. IN VIVO gp120 VACCINATION:

10 See figure 12 (mouse data):

Anti-gp120 ELISA Titers Elicited
by Secreted gp120*

15

	<u>Species</u>	<u>GMT (range)</u>
20	mouse (post 2 rounds, 200 μ g per round)	5,310 (1.8 x 10 ³ - 1.5 x 10 ⁴)
25	rabbit (post 3 rounds, 2 mg per round)	143 (75- 212)
	A.G. monkey (post 2 rounds, 2 mg per round)	171 (<10-420)

30 *Using V1Jns-tPA-gp120IIB as the inoculation vector, intramuscularly.